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(54) Title: NUCLEOTIDE EXPRESSION SYSTEMS WITH REDUCED IMMUNOGENICITY FOR USE IN GENE THERAPY

(57) Abstract

This invention suppresses the immune response to viral or non–viral based methods of gene delivery by incorporating immunosuppressive genes within the gene delivery system. These immunosuppressive genes may be used in addition to a therapeutic treatment or as a therapeutic treatment by themselves.

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NUCLEOTIDE EXPRESSION SYSTEMS WITH REDUCED IMMUNOGENICITY FOR USE IN GENE THERAPY

BACKGROUND OF THE INVENTION

The field of gene therapy has made significant gains in recent years. The combination of genetic defects being identified and gene target/delivery methods being developed has led to an explosion in the number of clinical gene therapy protocols. The central focus of gene therapy is to develop methods for introducing new genetic material into somatic cells. To date two general classes of gene transfer methods have evolved. The first is DNA-mediated gene transfer and involves direct administration of DNA to the patient in various formulations. These methods use genes as medicines in a manner much like conventional organic or protein compounds. DNA-mediated gene transfer however has proven quite difficult. Methodology such as micro-injection, lipofection, and receptor mediated endocytosis have usually resulted in lower gene transfer, and have usually established only transient residence of the novel gene in the targeted cell. Permanent incorporation of genes into cells occurs rarely after DNA-mediated gene transfer in cultured cells (less than 1×10^5 cells) and has not been significantly observed *in vivo*. Thus DNA-mediated gene transfer may be inherently limited to the use of genes as medicines that are administered by conventional parenteral routes to provide a therapeutic effect over predictable period of time. Studies of a therapeutic gene product may be constituted by repetitively dosing the patient with degenerate material much like conventional pharmaceutical medicines.

Viral gene transfer on the other hand involves construction of synthetic virus particles (vectors) that lack pathogenic functions. The virus particles are incapable of replication and contain a therapeutic or diagnostic gene within the viral genome which is delivered to cells by the process of infection. To date the viral vector which has achieved the most success is the retroviral vector. The prototype for a retroviral mediated gene transfer is a retroviral vector derived from Moloney Murine Leukemia Virus. Retroviral vectors have

several properties that make them useful for gene therapy. First is the ability to construct a "defective" virus particle that contains the therapeutic gene and is capable of infecting cells but lacks viral genes and expresses no viral gene products which helps to minimize host response to potential viral epitopes.

Retroviral vectors are capable of permanently integrating the genes they carry into the chromosomes of the target cell. Considerable experience in animal models and initial experience in clinical trials suggest that these vectors have a high margin of safety.

Vectors based on adenovirus have recently proven effective as vehicles for gene transfer *in vitro* and *in vivo* in several cell types. Adenoviral vectors are constructed using a deleted adenoviral genome that lacks either the e-3 gene region and/or the e-1 gene region that is required for producing a replicating adenovirus particle. Recombinant genes are inserted into the site of the deleted gene region(s). Adenoviral particles are then produced in a cell line that is able to express e-1 or e-3 genes and thus capable of assembling a viral particle which contains only the recombinant viral genome with the therapeutic gene.

Adenoviral vectors differ from retroviral vectors in that they do not integrate their genes into the target cell chromosome. Adenoviral vectors will infect a wide variety of both dividing and non-dividing cells *in vitro* and *in vivo* with a high level of efficiency providing expression of their recombinant gene for a period of several weeks to months.

Current technology has enabled construction of adenoviral vectors that are incapable of proliferating however they are not completely "defective" and will express a series of viral gene products which can generate host immune response to the viral epitopes presented causing quick elimination of the already transient vector. Adenoviral vectors remain capable of inducing cell lysis and an inflammatory response. Severe inflammation has been noticed during the experimental clinical trial for the treatment of cystic fibrosis.

Other viruses exhibit properties that may be useful as potential vectors for gene therapy. One such virus is the adeno-associated virus vector. It, like

the retrovirus can provide a completely defective vector that permanently integrates in the chromosome of the target cell. This adenoviral vector integrates at a predictable location within the affected cell and could make this type of vector safer than those that integrate randomly into the genome.

Another promising viral vector is based on the Herpes Simplex virus. Herpes virus vectors are capable of infecting cells and persisting indefinitely in a latent state. Traditionally the herpes simplex virus vector involves genetic engineering of the viral genome to render it useful for serial propagation and for sustained expression of foreign genes in a suitable host. Additional components may also be added such as the Epstein Barr virus nuclear antigen gene and latent origin of replication to maintain the vector in episomal state, as described in United States Patent Number 5,830,727.

Regardless of the method of delivery, an increasing problem in gene therapy protocols involves the host's immune response to the proteins encoded by the genes inserted. The majority of protocols rely on viral based gene delivery and thus face similar problems to those encountered by wild type virus. (Poller, W., et al., "Stabilization of transgene expression by incorporation of E3 region genes into an adenoviral factor IX vector and by transient anti-CD4 treatment of the host", *Gene Ther*, 3(6):521-30 (1996); Lochmuller, H., et al., "Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice", *Gene Ther*, 3(8):706-16 (1996); Tripathy, S.K., et al., "Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors", *Nat Med*, 2(5):545-50 (1996); Smith, T.A., et al., "Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector", *Gene Ther* 3(6):496-502 (1996); Riddell, S.R., et al., "T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients", *Nat Med* 2(2):216-23 (1996)). Even with the advent of techniques to design defective vectors which minimize the viral epitopes exposed to the immune

system, the host's immune system views the introduced therapeutic genes and their transcribed proteins as foreign and responds vigorously to eliminate the challenge. Once new or altered genes are delivered and expressed, the patient's immune system encounters protein epitopes to which it has not previously been exposed. The new epitopes stimulate the immune system to eliminate cells and/or viruses presenting them through a variety of means. Immune system response to gene therapy treatments has been documented or suspected in several gene therapy experiments and clinical trials(Koenig, S., *A lesson from the HIV patient: the immune response is still the bane (or promise) of gene therapy [comment].* Nat Med, 1996. 2(2):165-7; Riddell, S.R., *et al., T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients [see comments].* Nat Med, 1996. 2(2):216-23). The immune response can cause a rapid loss of expression of the therapeutic gene which will render most gene therapy protocols ineffective.

As can be seen a need exists in the art for a gene expression system which will minimize host immune response to the therapeutic gene allowing the foreign gene to persist without rapid elimination. This invention provides novel expression systems for use in nucleotide delivery vehicles which use sequences which encode immune suppressing agents which reduce host immune response to the therapeutic or diagnostic gene which is to be expressed.

It is an object of the present invention to provide nucleotide expression systems for therapeutic or for diagnostic purposes which have decreased immunogenicity.

It is yet another object of the invention to provide nucleotide expression systems which include immunosuppressive agents isolated from several viral genomes which allow the vehicle to evade the recipient cell immune response.

It is yet another object of the invention to provide a nucleotide expression system for permanent cell transformation with improved efficacy through longer persistent presence in recipient cells.

It is yet another object of the present invention to provide methods and protocols for use of the expression systems of the invention in design of nucleotide delivery vehicles and in the use of genetic engineering techniques for transformation of cells for in vivo, in vitro, and ex vivo gene therapy.

SUMMARY OF THE INVENTION

The present invention seeks to utilize genetic engineering techniques to provide a recombinant nucleotide expression system which provides for the continued persistence of introduced genes through immune suppression. In one aspect, the invention provides a recombinant expression system adapted to express immunosuppression genes isolated from virus genomes or functional equivalents or parts thereof, so that host immune response to the introduced therapeutic gene is minimized.

As used herein the term "immunosuppression gene" shall mean any nucleotide sequence, the expression of which provides a protein product which enables a virus to evade, diminish or suppress the immune system reaction of the recipient host to that virus. Such immune responses can include but are not limited to cytotoxic T lymphocyte response, MHC-1, MHC-2, T helper cell, cytokines, Interleukins, natural killer cells, neutrophils, macrophages, β-cell, plasma cells, tissue macrophages (e.g. Kuffer cell in liver) and dendritic cells. Examples of these genes include but are not limited to Epstein-Barr virus BHRF1, LMP-1 and LMP-2A, adenovirus E1B/19k and E1B/55k, cowpox virus crmA and CHOhr, baculovirus p35 and IAP, molluscum contagiosum virus MC159 and MCO66L, equine herpesvirus-2 E8, rabbitpox virus SPI-1, simian virus 40 T-Ag, papilloma virus E7, cytomegalovirus IE2, UL18, and US6, myxoma virus M-T5, MT-2, and M-T4, vaccinia virus E3L and K3L, and herpesvirus samurai Tip. Other examples of immunosuppression genes will be discussed throughout and may be isolated and identified in accordance with the teachings herein.

The term "expression system" is used herein to refer to a genetic sequence which includes a protein encoding region which is operably linked to

all of the genetic signals necessary to achieve expression of the protein encoding region. Traditionally, the expression system will include a regulatory element such as a promoter or enhancer, to increase transcription and/or translation of the protein encoding region, or to provide control over expression. The regulatory element may be located upstream or downstream of the protein encoding region, or may be located at an intron (noncoding portion) interrupting the protein encoding region. Alternatively it is also possible for the sequence of the protein encoding region itself to comprise regulatory ability.

The term "functional equivalent" refers to any derivative which is functionally substantially similar to the referenced sequence or protein. In particular the term "functional equivalent" includes derivatives in which nucleotide base(s) and/or amino acid(s) have been added, deleted or replaced without a significantly adverse effect on biological function and which will hybridize under high conditions of stringency according to protocols known in the art and disclosed in Maniantis et. al., "Molecular Cloning" cold Spring Harbor Press, (1989).

As used herein the term "therapeutic gene" shall be interpreted in include any nucleotide sequence, the expression of which is desired in a host cell. This can include any genetic engineering protocol for introduction of such sequence which would benefit from reduced immunogenicity to the introduced gene and includes antisense type strategies, diagnostic protocols, or gene therapy.

Thus the invention in one embodiment includes a recombinant expression system which comprises a therapeutic nucleotide sequence, the expression of which is desired in a cell and a recombinant nucleotide sequence which encodes an immunosuppression agent. The immunosuppression agent and nucleotide sequence may be operably linked to a single regulatory system to promote expression of both or may be separately incorporated into unique expression systems.

In a preferred embodiment the expression system of the invention is included within a gene delivery vehicle or vector for transformation of recipient cells. The immune suppression gene is one which is non-native to the vector of choice and preferably is maintained within a vector which has minimal viral encoded proteins. The invention is especially useful for transformation protocols which seek to achieve permanent integration and/or expression of gene products. The invention further encompasses vectors containing the recombinant expression systems defined above, cells transformed with such vectors, and genetic engineering protocols using these components.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the LXSN retroviral gene therapy vector.

Figures 2A and 2B are schematic representations of the cloning method and resulting vectors used. Figure 2A is the HSV ICP47 encoding gene was amplified from purified HSV DNA and ligated into a shuttle vector. The gene was sequenced to confirm its identity. The gene was then cloned into the EcoRI restriction site of the LXSN vector resulting in the LISN vector. Figure 2B is the hCMV US11 gene was cloned using a similar method from a hCMV infected cell line to obtain the LUSN vector.

Figures 3A, 3B and 3C are Northern blot analysis to confirm that the cell lines constructed were translating the expected RNA. Figure 3A is a 1.25% agarose gel electrophoresis separating 10 μg total RNA preparations from the four indicated cell lines. Figure 3B is the RNA was transferred to nylon membrane, probed with radiolabeled ICP 47 DNA, and detected by auto radiography. The band observed is the predicted size for the LISN RNA.

Figure 3C is the same nylon membrane probed with radiolabeled US11 DNA. The band observed is the predicted size of the LUSN RNA.

In Figure 4, relative MHC class I cell surface expression was determined using a anti MHC I monoclonal Ab and a FITC labeled secondary Ab. The results were analyzed by a fluorescence activated cell sorter (FACS), and the results are shown in Figure 4. A375 no Ab measures autofluorescence by the A375 cell line. A375 no primary was labeled with only the secondary Ab to determine if there was an appreciable level of non specific binding from the secondary Ab.

Figure 5 depicts the specific lysis of four target cell lines by TALL-104 CTL cells was determined over five effector/target cell ratios. The percent specific lysis was determined as described in methods.

Figures 6A-F depict cell surface MHC I inhibition. Transduced mixed populations of cells were labeled with anti-MHC I Ab (VMRD Inc.) at 0.1 μg/.5 x 10^6 cells at 37 degrees for 1 hour. Cells were washed with PBS 2x and a 1:150 dilution of goat anti-mouse FITC labeled secondary Ab (Sigma) was added for 1 hour at 37 degrees. Cells were again washed 2x and suspended in Phenol red free/serum free Opti-MEM® media for FACS analysis. 3 samples of each were averaged and the standard deviations determined. All cell lines were tested with no Ab, no primary, nontransduced controls, and LXSN transduced controls. 6A (Transduced 293 Cell Lines) is a transformed human kidney epithelial cell line. 6B (Transduced VA13 Cell Lines) is a transformed human fibroblast cell line. 6C (Transduced A549 Cell Lines) is a human lung carcinoma cell line. 6D (Transduced A375 Cell Lines, and this is a repeat of the experiment described in Fig. 4) is a human melanoma cell line. 6E (Transduced Ovcar Cell Lines) is a human ovarian carcinoma cell line. 6F (Transduced IGROV Cell Lines) is a human ovarian carcinoma cell line. Error bars represent the standard error of the mean from three trials.

Figures 7A and 7B depict specific Lysis of transduced target cells by TALL-104 effector cells. $5x10^3$ target cells/well are aliquoted on a 96 well tissue culture plate (4 wells/data point). Effector cells are added at the indicated ratios and incubated for 6 hours. 100% lysis is determined by completely lysing a set of control wells. Cell lysis is determined by measuring the amount of a stable cytosolic enzyme (lactate dehydrogenase, LDH) released into the well and using a color reaction (Promega Corp. Cytotox 96® assay kit). (Sinensky, M.C., A.L. Leiser, and H. Babich, "Oxidative stress aspects of the cytotoxicity of carbamide peroxide: in vitro studies", *Toxicol Lett*, 75(1-3):101-9 (1995). Baseline LDH levels are established and spontaneous LDH release by effector and target cells is subtracted. Extent of color reaction is determined using a 96 well plate spectrophotometer at 92 nm. 7A TALL-104 specific lysis of A375 control and transduced cell lines. This is the exact same data (Fig. 7A) as in Fig. 5. 7B TALL-104 specific lysis of VA13 control and transduced cell lines.

Figure 8 depicts a schematic diagram of the LISH vector. The structure of LISH where the main difference from LISN is the substitution of the hygromycin resistance gene in place of the neomycin resistance gene.

Figure 9 depicts a schematic diagram of the LUISN vector. The structure of LIUSN, where ICP 47 and US11 are expressed from the same RNA molecule using an internal ribosome entry site IRES. This construct minimizes the number of controls needed to assess MHC 1 levels and specific lysis.

Figures 10A and 10B depict ICP 47 and US11 function in dog and rat cells. The dog bone marrow cell line D17 A), and the rat glioblastoma cell line 9L B), were transduced and assayed for cell surface MHC I as previously described. ICP 47 does not function in rodent cells but does function in dog cells. US11 functions in both rodent and dog cells.

Figure 11 depicts Lymphocyte expression vectors. The IRES immune suppression cassettes will be constructed and inserted into LGSN vector. The LGSN vector was constructed by subcloning the humanized, red shifted eGFP (Clonetech Inc.) into the LXSN vector.

Figure 12 depicts a schematic representation of LαSI/N and LαSU/N vectors. Both vectors will be tested for immune suppression as previously described and α-ID expression by western analysis.

Figure 13 is a map of the pLUSN vector which was designed according to the present invention.

Figure 14 is the nucleotide sequence of the pLUSN vector (SEQ ID NO: 2).

Figure 15 is a map of the pLXSU-IRES-N vector which was designed according to the present invention.

Figure 16 is the nucleotide sequence of the pLXSU-IRES-N vector (SEQ ID NO: 5).

Figure 17 is a map of the pLISN vector which was designed according to the present invention.

Figure 18 is the nucleotide sequence of the pLISN vector (SEQ ID NO: 1).

Figure 19 is a map of the pLXSI-IRES-N vector which was designed according to the present invention.

Figure 20 is the nucleotide sequence of the pLXSI-IRES-N vector (SEQ ID NO: 3).

Figure 21 is the nucleotide sequence of the pLXSN vector (SEQ ID NO:4).

DETAILED DESCRIPTION OF THE INVENTION

It has long been known that viruses have evolved means to suppress specific aspects of the immune response (McFadden, G. and K. Kane, *How DNA viruses perturb functional MHC expression to alter immune recognition.* Adv Cancer Res, 1994. **63**:117-209). Recently, several viral genes have been identified and characterized that disrupt specific steps in the host's detection and elimination of the given virus. These findings have been of keen interest to researchers seeking to aid the immune system in combating potentially damaging viral infections. The nucleotide sequences of these genes have also been characterized and are known and accessible to those of skill in the art. Some examples include but are not limited to the Herpes Simplex Virus (HSV) type 1 ICP-47 encoding gene York, I.A., *et al., A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes.* Cell, 1994. **77**(4):525-35; Rosenthal, K.L., *et al., Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes.* J Virol, 1987. **61**(8):2438-47) and the human Cytomegalovirus (hCMV) US11 gene Jones, T.R., *et al., Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains.* J Virol, 1995. **69**(8):4830-41; Wiertz, E.J., *et al., The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol.* Cell, 1996. **84**(5):769-79).

The expression system of the invention in its simplest context comprises an expression system including a therapeutic gene or nucleotide sequence the expression of which is desired in a recipient cell and a recombinant immune suppression gene or expression of recombinant immune suppression gene alone. Any nucleotide sequence as defined supra can be used in the expression system of the invention. Similarly, any of several known and yet to be

discovered immunosuppression genes may also be used. Examples of such genes include Epstein-Barr virus BHRF1, LMP-1 and LMP-2A, adenovirus E1B/19k and E1B/55k, cowpox virus crmA and CHOhr, baculovirus p35 and IAP, molluscum contagiosum virus MC159 and MCO66L, equine herpesvirus-2 E8, rabbitpox virus SPI-1, simian virus 40 T-Ag, papilloma virus E7, cytomegalovirus IE2, UL18, and US6, myxoma virus M-T5, MT-2, and M-T4, vaccinia virus E3L and K3L, and herpesvirus samurai Tip. The nucleotide sequences of these genes are generally known and accessible to those of skill in the art from information sources such as GenBank. Further, methods for identification, isolation, and sequencing of other immunosuppression genes are disclosed in the articles incorporated by reference herein and may be used to identify and sequence yet other immunosuppression genes to be used in accordance with the present invention.

In yet another embodiment, the use of multiple immune suppression genes, either transcriptionally linked by internal ribosome entry sites (IRES) or individually controlled by separate promoters, in a single gene therapy vector promises to increase the effectiveness of this approach (Parks, G.D., G.M. Duke, and A.C. Palmenberg, "Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5' noncoding sequences to the P3 region", *J Virol*, 1986. **60**(2):376-84.

The nucleotide expression system of the invention as well as methods of the invention employing genetic engineering techniques using this system have the benefit of reducing, inhibiting or evading the immune response generated to the therapeutic gene thus increasing survival time. Virtually all conventional gene therapy protocols can benefit from increased survival time of the therapeutic gene and would therefore benefit by inclusion of these immune suppression genes in transformation vehicles. Alternatively, expression of recombinant immune suppression genes would be expressed to reduce tissue or cell allograft rejection.

In a preferred embodiment the nucleotide expression system of the invention is included within an appropriate gene transfer vehicle which is then

used to transduce cells to express the gene of interest and to reduce any immune response to the gene by the recipient host cells. The gene delivery vehicle can be any delivery vehicle known in the art and can include simply naked DNA which is facilitated by a receptor mediated transfection as well as any of a number of vectors. Such vectors include but are not limited to eukaryotic vectors, prokaryotic vectors (such as for example bacterial vectors) and viral vectors including but not limited to retroviral vectors, adenoviral vectors, adeno-associated viral vectors, lentivirus vectors (human and other including porcine), Herpes virus vectors, Epstein-Barr virus vectors, SV40 virus vectors, pox virus vectors, pseudotype virus vectors.

This invention is particularly useful for viral vector transformation strategies which seek to accomplish permanent as opposed to transient expression of the therapeutic gene. As such the invention is particularly suited to use of vectors such as retroviral, adeno-associated, or lenti viral vectors which integrate into host DNA. The invention is also particularly suited to vector systems which persist indefinitely in transformed cells such as HSV vectors or those which are maintained in episomal state such as by use of Epstein Barr genetic elements.

The invention is also particularly useful with vectors which are defective and thus contain no or minimal amounts of viral epitopes thus minimizing the immune reaction generated to the vector itself or vector components. These vectors may be further optimized by the present invention to minimize any immune response to the therapeutic gene itself.

Any of a number of standard gene delivery transformation methods can be used such as lipid mediated transfection, receptor mediated transfection, calcium phosphate transfection, electroporation particle bombardment, naked-direct DNA injection, diethylaminoethyl (DEAE-dextran transfection).

The expression vehicles (vectors) of the invention can be engineered by any of a number of techniques known to those of skill in the art. The following is a summary of techniques for construction and transformation of the vectors of the invention.

GENETIC ENGINEERING TECHNIQUES FOR CONSTRUCTION AND DELIVERY OF VECTORS

Any of a number of standard gene delivery transformation methods can be used for the invention including lipid mediated transfection, receptor mediated transfection, calcium phosphate transfection, electroporation particle bombardment, naked-direct DNA injection, diethylaminoethyl (DEAE-dextran transfection).

In a preferred embodiment the expression vehicles or vectors of the invention comprising the expression system also comprise a selectable marker gene to select for transformants as well as a method for selecting those transformants for propagation of the construct in bacteria. Such selectable marker may contain an antibiotic resistance gene, such as those that confer resistance to ampicillin, kanamycin, tetracycline, or streptomycin and the like. These can include genes from prokaryotic or eukaryotic cells such as dihydrofolate reductase or multi-drug resistance I gene, hygromycin B resistance that provide for positive selection. Any type of positive selector marker can be used such as neomycin or Zeosyn and these types of selectors are generally known in the art. Several procedures for insertion and deletion of genes are known to those of skill in the art and are disclosed. For example in Maniantis, "Molecular Cloning", Cold Spring Harbor Press. See also Post et al., *Cell*, Vol. 24:555-565 (1981). An entire expression system must be provided for the selectable marker genes and the genes must be flanked on one end or the other with promoter regulatory region and on the other with transcription termination signal (polyadenylation cite). Any known promoter/transcription termination combination can be used with the selectable marker genes. For example SV40 promoter and SV40 poly A.

A therapeutic gene to be expressed can then be introduced into the vector of the invention. The foreign DNA can comprise an entire transcription unit, promoter-gene-poly A or the vector can be engineered to contain promoter/transcription termination sequences such that only the gene of interest need be inserted. These types of control sequences are known in the

art and include promoters for transcription initiation, optionally with an operator along with ribosome binding site sequences. Examples of such systems include beta-lactase (penicillinase) and lactose promoter systems, (Chang et al., *Nature*, 1977, 198:1056); the Tryptophan (trp) promoter system (Goeddel, et al., *Nucleic Acid Res.*, 1980, 8:4057) and the lambda derived Pl promoter and N-gene ribosome binding site (Shimatake et al., *Nature* 1981, 292:128). Other promoters such as cytomegalovirus promoter or Rous Sarcoma Virus can be used in combination with various ribosome elements such as SV40 poly A. The promoter can be any promoter known in the art including constitutive, (supra) inducible, (tetracycline-controlled transactivator (tTA)-responsive promoter (tet system, Paulus, W. et al., "Self-Contained, Tetracycline-Regulated Retroviral Vector System for Gene Delivery to Mammalian Cells", J of Virology, Jan. 1996, Vol. 70, No. 1, pp. 62-67)),or tissue specific, (such as those cited in Costa, et. Al., European journal of Biochemistry, 258 "Transcriptional Regulation Of The Tissue-Type Plasminogen Activator Gene In Human Endothelial Cells: Identification Of Nuclear Factors That Recognize Functional Elements In The Tissue-Type Plasminogen Activator Gene Promoter" pgs, 123-131 (1998); Fleischmann, M., et. Al., FEBS Letters 440 "Cardiac Specific Expression Of The Green Fluorescent Protein During Early Murine Embryonic Development" pgs. 370-376, (1998); Fassati, Ariberto, et. Al., Human Gene Therapy, (9:2459-2468) "Insertion Of Two Independent Enhancers In The Long Terminal Repeat Of A Self Inactivating Vector Results In High-Titer Retroviral Vectors With Tissue Specific Expression" (1998); Valerie, Jerome, et. Al. Human Gene Therapy 9:2653-2659, "Tissue Specific Cell Cycle Regulated Chimeric Transcription Factors For The Targeting Of Gene Expression To Tumor Cells, (1998); Takehito, Igarashi, et. Al., Human Gene Therapy 9:2691-2698, "A Novel Strategy Of Cell Targeting Based On Tissue-Specific Expression Of The Ecotropic Retrovirus Receptor Gene", 1998; Lidberg, Ulf et.al. The Journal of Biological Chemistry 273, No.47, "Transcriptional Regulation Of The Human Carboxyl Ester Lipase Gene In Exocrine Pancreas" 1998; Yu, Geng-Sheng et.

Al., The Journal of Biological Chemistry 273 No. 49, "Co-Regulation Of Tissue-Specific Alternative Human Carnitine Palmitoyltransferase IB Gene Promoters By Fatty Acid Enzyme Substrate" (1998)). These types of sequences are well known in the art and are commercially available through several sources, ATCC, Pharmacia, Invitrogen, Stratagene, Promega.

In a most preferred embodiment the vector comprises a specifically engineered multi-cloning site within which several unique restriction sites are created. Restriction enzymes and their cleavage sites are well known to those of skill in the art.

In a preferred embodiment, a packaging cell line is transduced with a viral vector containing the therapeutic nucleotide sequence to form a producer cell line including the viral vector. The producer cells may then be directly administered, whereby the producer cells generate viral particles capable of transducing the recipient cells.

In a preferred embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus.

Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the therapeutic gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

These new genes may be incorporated into the proviral backbone in several general ways. The most straightforward constructions are ones in

which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter.

Efforts have been directed at minimizing the viral component of the viral backbone, largely in an effort to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

In one embodiment, the retroviral vector may be one of a series of vectors described in Bender, et al., *J. Virol.* 61:1639-1649 (1987), based on the N2 vector (Armentano, et al., *J. Virol.*, 61:1647-1650) containing a series of deletions and substitutions to reduce to an absolute minimum the homology between the vector and packaging systems. These changes have also reduced the likelihood that viral proteins would be expressed. In the first of these vectors, LNL-XHC, there was altered, by site-directed mutagenesis, the natural ATG start codon of gag to TAG, thereby eliminating unintended protein synthesis from that point.

In Moloney murine leukemia virus (MoMuLV), 5' to the authentic gag start, an open reading frame exists which permits expression of another glycosylated protein ($pPr80^{gag}$). Moloney murine sarcoma virus (MoMuSV) has alterations in this 5' region, including a frameshift and loss of glycosylation sites, which obviate potential expression of the amino terminus of $pPr80^{gag}$. Therefore, the vector LNL6 was made, which incorporated both the altered ATG of LNL-XHC and the 5' portion of MoMuSV. The 5' structure of the LN vector series thus eliminates the possibility of expression of retroviral reading frames, with the subsequent production of viral antigens in genetically transduced target cells. In a final alteration to reduce overlap with

packaging-defective helper virus, Miller has eliminated extra env sequences immediately preceding the 3' LTR in the LN vector (Miller, et al., *Biotechniques*, 7:980-990, 1989).

The paramount need that must be satisfied by any gene transfer system for its application to gene therapy is safety. Safety is derived from the combination of vector genome structure together with the packaging system that is utilized for production of the infectious vector. Miller, et al. have developed the combination of the pPAM3 plasmid (the packaging-defective helper genome) for expression of retroviral structural proteins together with the LN vector series to make a vector packaging system where the generation of recombinant wild-type retrovirus is reduced to a minimum through the elimination of nearly all sites of recombination between the vector genome and the packaging-defective helper genome (i.e. LN with pPAM3).

In one embodiment, the retroviral vector may be a Moloney Murine Leukemia Virus of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al. (1987) and Miller, et al. (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragment or truncations thereof, are not expressed.

In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral vector includes each of these cloning sites.

When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from

the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., *Biotechniques*, 7:(9):980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vector then is employed to transduce a packaging cell line to form a producer cell line. Examples of packaging cells which may be transfected include, but are not limited to the PE501, PA317, Ψ2, Ψ-AM, PA12, T19-14X, VT-19-17-H2, ΨCRE, ΨCRIP, GP+E-86, GP+envAM12, and DAN cell lines. The vector containing the therapeutic nucleic acid sequence may transduce the

packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation.

The producer cells then are administered directly to or adjacent to desired recipient cells.

In a preferred embodiment the invention comprises a viral vector which commonly infects humans and packaging cell line which is human based. For example vectors derived from viruses which commonly infect humans such as Herpes Virus, Epstein Barr Virus, may be used which do not express an active α-galactosyl envelope.

In a most preferred embodiment the vector comprises a Herpes Simplex Virus plasmid vector. Herpes simplex virus type-1 (HSV-1) has been demonstrated as a potential useful gene delivery vector system for gene therapy, Glorioso, J.C., "Development of Herpes Simplex Virus Vectors for Gene Transfer to the Central Nervous System. Gene Therapeutics: Methods and Applications of Direct Gene Transfer", Jon A. Wolff, Editor, 1994 Birkhauser Boston, 281-302; Kennedy, P.G., "The Use of Herpes Simplex Virus Vectors for Gene Therapy in Neurological Diseases", Q J Med, Nov. 1993, 86(11):697-702; Latchman, D.S., "Herpes Simplex Virus Vectors for Gene Therapy", *Mol Biotechnol*, Oct. 1994, 2(2):179-95.

HSV-1 vectors have been used for transfer of genes to muscle. Huard, J., "Herpes Simplex Virus Type 1 Vector Mediated Gene Transfer to Muscle", *Gene Therapy*, 1995, 2, 385-392; and brain, Kaplitt, M.G., "Preproenkephalin Promoter Yields Region-Specific and Long-Term Expression in Adult Brain After Direct *In Vivo* Gene Transfer Via a Defective Herpes Simplex Viral Vector", *Proc Natl Acad Sci USA*, Sep 13, 1994, 91(19):8979-83, and have been used for murine brain tumor treatment, Boviatsis, E.J., "Long-Term Survival of Rats Harboring Brain Neoplasms Treated With Ganciclovir and a Herpes Simplex Virus Vector That Retains an Intact Thymidine Kinase Gene", *Cancer Res*, Nov 15, 1994, 54(22):5745-51; Mineta, T., "Treatment of Malignant

Gliomas Using Ganciclovir-Hypersensitive, Ribonucleotide Reductase-Deficient Herpes Simplex Viral Mutant", *Cancer Res*, Aug 1, 1994, 54(15):3963-6.

Helper virus dependent mini-viral vectors have been developed for easier operation and their capacity for larger insertion (up to 140 kb), Geller, Al, "An Efficient Deletion Mutant Packaging System for Defective Herpes Simplex Virus Vectors: Potential Applications to Human Gene Therapy and Neuronal Physiology", *Proc Natl Acad Sci USA*, Nov 1990, 87(22):8950-4; Frenkel, N., "The Herpes Simplex Virus Amplicon: A Versatile Defective Virus Vector", *Gene Therapy. 1. Supplement 1*, 1994. Replication incompetent HSV amplicons have been constructed in the art, one example is the pHSVlac vector by Geller et al, *Science*, 241, Sept. 1988, incorporated herein by reference. These HSV amplicons contain large deletions of the HSV genome to provide space for insertion of exogenous DNA. Typically they comprise the HSV-1 packaging site, the HSV-1 "ori S" replication site and the IE 4/5 promoter sequence. These virions are dependent on a helper virus for propagation.

Primarily two types of mutant helper viruses have been developed to minimize recombination. Other complementary HSV helper virus systems are contemplated herein and are within the scope of those of skill in the art. One such system which has been developed is a temperature-sensitive mutant. An HSV temperature-sensitive (TS) mutant has been developed with a TS mutation in the IE3 gene. Davison et al, 1984, *J. Gen. Virol.*, 65:859-863. Consequently this virus has an IE phenotype, does not replicate DNA, does not significantly alter cellular physiology, and does not produce progeny virus at 37°C. Virus is grown at the permissive temperature of 37°C. TS mutants however have had a tendency to revert to wild type.

In contrast a second helper virus system is a deletion mutant with the majority of the IE3 gene simply deleted. These do not revert to wild type. Therefore HSV-1 vectors packaged using a deletion mutant as helper virus is the most preferred helper virus of the invention. See for example Patterson et al., 1990, *J. Gen. Virol.*, 71:1775-1783. Other replication incompetent helper viruses can be used and one of skill in the art will appreciate that other

mutations in the IE genes or other genes which result in a replication incompetent helper virus which will provide the appropriate replication and expression functions and which are coordinated with the helper cell line and vector are contemplated within this invention. Any cell line can be used for this step so long as it is capable of expressing the IE3 or replication dependent gene, or obtaining a helper cell line which has already been transformed and is commercially available. Any cell line can be used by introducing pHE and the plasmid containing the IE3 gene simultaneously. Next, the vector is delivered to the helper cell line by electroporation, calcium phosphate DNA transfection or any other suitable method. Any cell line can be used by introducing pHE and the plasmid containing the IE3 gene simultaneously. The cells are next infected with a helper virus IE3 deletion mutant or other corresponding deletion mutant which is replication incompetent. The IE3 gene or other such gene in the helper cell line complements the helper virus resulting in a productive HSV-1 infection and the resulting virus stock consists of HSV-1 particles containing either vector DNA or helper virus DNA, all of which are replication incompetent. Further information about helper cell lines and the methodology is disclosed in Geller et al., *PNAS*, 87:8950-8954, November 1990, "An Efficient Deletion Mutant Packaging System for Defective Herpes Simplex Virus Vectors: Potential Applications to Human Gene Therapy and Neuronal Physiology". The invention comprises a HSV mini vector which combines a replication incompetent HSV amplicon with other viral sequences such as those from Epstein-Barr virus, human papillomavirus, or bovine papillomavirus type 1 which allow the vector to be maintained in the cell in episomal form achieving a 10 times greater titer, and a very large DNA insert capacity.

One embodiment of the present invention involves a helper virus-dependent mini-viral vector comprising: (a) the HSV-1 "a" sequence for the package/cleavage signal and an "ori S" replication origin for the replication packaging of the plasmid (in response to signals to replicate and package from the helper virus); (b) an Epstein-Barr virus (EBV) nuclear antigen (EBNA-1)

gene and an EBV latent origin of replication (ori P) which allow the vector to be maintained in episomal form within the nucleus for replication without integration to the host genome and for even replication into each of two dividing cells; preferably (c) genes from prokaryotic cells for propagation of the vector in *E. coli* (a selectable marker gene such as the ampicillin resistance or tetracycline resistance gene and the *col.* E1 *ori*) and (d) a sequence encoding a immune suppression protein such as US11. Optionally the vector may also comprise prokaryotic genes that provide for a second selectable marker such as the genes for positive Hygromycin selection.

In this particular embodiment the packaging function of mini-vector DNA into Herpes simplex viral capsids is provided by a helper virus and a helper cell line.

In yet another embodiment the HSV vector can be engineered to produce a helper free viral vector as in Mann et al., "Construction of a Retro-Virus Packaging Mutant and its Use to Produce Helper-Free Defective Retrovirus", 33 Sal., p. 153-159, May 1983, Journal of Virology, September 1989, pp. 3822-3829, September 1989; Samulski "Helper Free Stocks of Recombinant Adeno-Associated Viruses: Normal Integration Does Not Require Viral Gene Expression"; and Kohn et al., "High Efficiency Gene Transfer Into Mammalian Cells: Generation of Helper-Free Recombinant Retrovirus With Broad Mammalian Host Range", PNAS, 81:6349-6353, October 1984. See also Okasinki, U.S. Patent No. 4,970,155 "HSV HELPER VIRUS INDEPENDENT VECTOR", incorporated herein by reference.

The expression system and transformation vehicle of the present invention can be used for any diagnostic or therapeutic genetic engineering protocol including *in vitro*, *ex vivo*, or *in vivo* expression of a desired nucleotide sequence. For example the expression vehicles of the invention can be used in any of a number of therapeutic treatment protocols in the treatment of cancer such as by the Herpes simplex virus, thymidine kinase gene transfer system Martuza RL et al., "Experimental therapy of human glioma by means of a genetically engineered virus mutant", Science, 1991; 252:854-856). Also in ex

vivo gene therapy protocols such as bone marrow purging (Seth P., et al., "Adenovirus-mediated gene transfer to human breast tumor cells: an approach for cancer gene therapy and bone marrow purging", *Cancer Res.* **56(6)**: 1346-1351 (1996; Andersen, N.S., et al., "Failure of immunologic purging in mantle cell lymphoma assessed by polymerase chain reaction detection of minimal residual disease", Blood, **90(**10:4212-4221 (1997)) thus when the transformed cells are reintroduced to the patient they will generate a decreased immune response. These may also be used for diagnostic purposes as well.

In a more preferred embodiment the present invention provides nucleotide expression vehicles (pLXSI-IRES-N, pLISN, pLXSU-IRES-N, pLGSN, pLISH, pLUISN, and pLUSN) constructed in accordance with the teachings herein and comprising a polynucleotide having a sequence substantially as set out in SEQ ID NO:1, 2, 3, or 4 or a portion or functional equivalent thereof.

In addition to genetic engineering protocols based upon delivery of a therapeutic gene, in yet another embodiment of the invention the immune suppression gene may itself by the therapeutic gene to be introduced to cells. Delivered on their own, these immunosuppression expression systems can be used as therapeutic agents in a number of protocols.

For example, several classes of viruses have evolved immunosuppression gene whose protein products specifically suppress the CTL response. (McFadden, G. and K. Kane, "How DNA viruses perturb functional MHC expression to alter immune recognition", *Adv Cancer Res* **63**:117-209 (2994); Fruh, K., et al., "A viral inhibitor of peptide transporters for antigen presentation", *Nature* **375**:6530):415-8 (1995); Wold, W.S. and L.R. Gooding, "Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions", *Virology*, 184(1):1-8 (1991); Beersma, M.F., M.J. Bijlmakers, and H.L. Ploegh, "Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains", *J Immunol*, **151**(9):4455-64 (1993)). Two such genes include the Herpes Simplex Virus (HSV) type 1 ICP-47 encoding gene, York, I.A., *et al.*, *A*

cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell, 1994. 77(4):525-35; Rosenthal, K.L., *et al., Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes.* J Virol, 1987. 61(8):2438-47) and the human Cytomegalovirus (hCMV) US11 gene Jones, T.R., *et al., Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains.* J Virol, 1995. 69(8):4830-41; Wiertz, E.J., *et al., The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol.* Cell, 1996. 84(5):769-79).

The CTL response has been identified as a contributing factor in many autoimmune diseases including; Crohn's, diabetes, and allergic reactions (Harris, S.J., *et al., Prediction of murine MHC class I epitopes in a major house dust mite allergen and induction of T1-type CD8+ T cell responses.* Int Immunol, 1997. 9(2):273-80; Nagata, M., *et al., Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice.* J Immunol, 1989. 143(4):1155-62).

According to the invention the immune suppression expression system of the invention may be administered as therapeutic agents to inhibit the CTL immune response associated with these diseases. The expression system again either alone or in an expression vehicle is administered to the patient in an amount effective for treatment of or reduction in symptoms of the diseases determined by one of skill in the art.

Additional autoimmune diseases that are potential targets for this therapy including; autoimmune anemias or cytopenias, ulcerative colitis, vascular heart disease, pericardial disease, vasculitis, hypersensitivity pneumonitis, interstitial lung diseases, cardiomyopathies, glomerulopathies, hepatitis/cirrhosis, acute/chronic pancreatitis, foreign body reactions, pulmonary inflammatory syndrome, osteoarthritis, psoriasis, sarcoidosis, vasculitis, Beech's syndrome, siogronssya, polymyositis, dermatomyositis,

systemic sclerosis, disease of immediate type hypersensitivity, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, eosinophilic pneumonias, myasthenia gravis, neuromuscular junction diseases, demyelinating diseases, polychondritis, infectious arthritis, arthritis due to deposition of calcium crystals, osteoarthritis, undifferentiated spondyloarthropathy, Sjogren's syndrome, immunologically mediated skin diseases, acute disseminated encephalomyelitis, acute hemorrhagic leukoencephalitis, muscular dystrophies, glomerulopathies, inflammatory bowel diseases, valvular heart diseases, cor pulmonale, and myocarditises. General suppression of MHC I expression by cells in the affected tissues should reduce or eliminate the problematic immune response. Additionally, organ transplants could greatly benefit from localized immune suppression such as these genes offer (Qin, L., *et al.*, "*Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival*". J Immunol, 1996. **156**(6):2316-23).

In yet another embodiment, successful expression of these genes in a transplant organ would reduce or eliminate the need to provide the patient with systemic immune suppression and its related problems.

In yet a further embodiment the immune suppression system of the invention may be used as a therapeutic agent in a cancer treatment protocol. According to this embodiment the immune suppression system of the invention includes multiple immune suppression agents which are expressed at high levels. At high levels these agents are become targeted by the immune system and the immune system targets and destroys transduced cells. As such the system may be used to transduce tumor cells to generate a immune response to these cells and to kill the cells. Leong, C.C., et al., "Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog", *J. Exp. Med.*, **188(3)**:614 (1998); Yokoyama, W.M., "Natural killer cell receptors", *Current Opinion in Immunology*, **10(3)**:298-305 (1998); Komatsu, F., et al., "Relationship between antibody-dependent cell-mediated

cytotoxicity due to anti-HTLV-1 and negative signal of major histocompatibility complex class I antigens on adult T-cell leukemia cell lines", *Oncology Research*, **10(2)**:59-67 (1998); Fletcher, J.M., et al., "Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA", *Journal of Immunology*, **161(5)**:2365-2374 (1998).

For this embodiment the expression system will include an inducible or preferably a tumor specific promoter to direct expression of the suicide genes to a tumor cell. Such promoters are known and accessible to those of skill in the art and include those listed in the following articles: Gotoh, et.al., *The Journal of Urology*, Vol. 160 "Development of Prostate-specific antigen promoter-based Gene Therapy for Androgen-Independent Human Prostate Cancer" pgs. 220-229 1998; Pang, Shen et. al, *Cancer Research* Vol 57 "Identification of A Positive Regulatory Element Responsible for Tissue-specific Expression of Prostate-specific Antigen" page 495-499, (1997); Lin, Ching-Shwun, et. Al, *The Journal of Biological Chemistry*, Vol 268 No. 4 "Characterization of the Human L-Plastin Gene Promoter in Normal and Neoplastic Cells" pgs 2793-2801(1993); and Lin, Ching-Shwun et. Al., DNA and Cell Biology, volume 16 No. 1 "The Murine L-Plastin Gene Promoter: Identification and Comparison with the Human L-plastin Gene Promoter" pgs. 9-16 (1997)

To fully exploit the benefits of the methods and compositions described herein, the use of many general gene therapy improvements are contemplated and are intended to be within the scope of this invention. In this manner, improvements as higher viral titer production, more efficient gene delivery, and targeted gene delivery will be utilized, and are intended to be within the scope of the invention.

The selection and use of additional components to improve gene therapy vectors are seen as simply characterized through routine experimentation and are intended to be within the scope of this invention.

All references cited herein are hereby expressly incorporated in their entirety be reference including the following:

Miller, et al., "Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production", *Molecular and Cellular Biology*, 6:(8), 2895-2902 (1986)

Koenig, Scott, "A lesson from the HIV patient: The immune response is still the bane (or promise) of gene therapy", *Nature Medicine*, 2:(6), 165 (1996)

York, et al., "A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes", *Cell*, 11: 525-535 (1994)

Rosenthal, K., "Cells Expressing Herpes Simplex Virus Glycoprotein gC byt not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes", *Journal of Virology*, 61(8) 2438-2447 (1987)

Jones, et al., "Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex Class I heavy chains", *Journal of Virology*, 69(8) 4830-4841

Wiertz, et al., "The human cytomegalovirus US11 gene product dislocates MHC Class 1 heavy chains from the endoplasmic reticulum to the cytosol", *Cell*, 84 769-779

Hill, et al., "Herpes simplex virus turns off the TAP to evade host immunity", *Nature*, 375 411-415 (1995)

Galocha et al., "The active site of ICP47, a hespes simplex virus-encoded inhibitor of the major histocompatibility complex (MHC)-encoded peptide transporter associated with Antigen Processing (TAP), maps to the NH_2-terminal 35 residues", 185(9) 1565-1572 (1997)

Tomazin et al., "Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP", *EMBO J* 15(13) 3256-3266 (1996)

Kwangseog et al., "Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47", *EMBO J* 15(13) 3247-3255 (1996)

Früh et al., "A viral inhibitor of peptide transporter for antigen presentation", *Nature*, **375** 415-418 (1995)

Machold et al., "The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complecx (MHC Class I heavy chains", *J Exp. Med.*, **185(2)** 363-366 (1997)

Jones et al., "Human cytomegalovirus US2 destabbilizes major histocompatibility complex Class I heavy chains", *Journal of Virology*, **71(2)** 2970-2979 (1997)

Nagata et al., "Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice", *Journal of Immunology*, **143**, 1155-1162 (1978)

Qin et al., "Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival", *The Journal of Immunology*, **156** 2316-2323 (1996)

Parks, et al., "Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5' noncoding sequences to the P3 region", *Journal of Virology* **60(2)** 376-384 (1986)

Früh et al., "A viral inhibitor of peptide transporters for antigen presentation", *Nature*, **375** 415-418 (1995)

The following examples are intended to further illustrate the compositions and methods of the invention and are intended to limit the invention in no way.

EXAMPLES

EXAMPLE 1

Of the many types of immune responses, the CTL cytotoxic T lymphocyte (CTL) response to MHC I restricted antigens is known to play an early and key role in host response to viral infection. As expected, host CTL response to transgenes and viral proteins has been observed in viral based

gene therapy protocols. Host elimination of viral vectors, (Lochmuller, H., et al., "Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice", *Gene Ther*, 3(8):706-16 (1996); Smith, T.A., et al., "Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector", *Gene Ther* 3(6):496-502 (1996); Riddell, S.R., et al., "T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients", *Nat Med* 2(2):216-23 (1996)) and specific T cell response to a transgene (Poller, W., et al., "Stabilization of transgene expression by incorporation of E3 region genes into an adenoviral factor IX vector and by transient anti-CD4 treatment of the host", *Gene Ther*, 3(6):521-30 (1996); Tripathy, S.K., et al., "Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors", *Nat Med*, 2(5):545-50 (1996)) have been well documented. Therefore, overcoming host CTL response and prolonging vector survival is now recognized as a high priority goal in gene therapy systems.

In the normal pathway, MHC I molecules are synthesized in the lumen of the endoplasmic reticulum (ER) and become fully mature. Mature MHC I molecules are transported through the golgi apparatus and presented on the cell surface. Unassembled MHC I molecules are degraded in the golgi and are not presented on the cell surface. (McFadden, G. and K. Kane, "How DNA viruses perturb functional MHC expression to alter immune recognition", *Adv Cancer Res* 63:117-209 (1993)). The 8-10 amino acid peptides which determine the antigenic epitopes are generated from foreign proteins in the cytosol mainly by proteosomes and are transported into the ER by the transporter associated with antigen processing (TAP) peptide transporter. (Heemels, M.T., et al., "Peptide translocation by variants of the transporter associated with antigen processing", *Science*, 262(5142):2059-63 (1993)). The immune suppression agent ICP 47 binds to the peptide loading site on the cytosolic side

of the TAP transporter. (Tomazin, R., et al., "Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP", *EMBO J*, 15(13):3256-66 (1996). This ICP 47/TAP association prevents the 8-10 amino acid viral peptides from entering the ER and thus reduces the number of MHC I molecules present on the cell surface. The hCMV US11 gene product affects a different step in the antigen processing and presentation pathway. The US11 gene encodes a transmembrane glycoprotein which translocates mature MHC I molecules from the ER back to the cytosol where they are rapidly degraded. (Beersma, M.F., M.J. Bijlmakers, and H.L. Ploegh, "Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains", *J Immunol*, 151(9):4455-64 (1993). In cells where US11 is expressed, newly synthesized MHC I has a half-life of less than 1 minute and is not presented on the cell surface. These viral proteins are small and easily fit within the packaging size constraints of retroviral gene therapy vectors.

For these experiments, the murine derived retroviral vector LXSN was used to deliver and express the viral immune suppression genes. (Miller, A.D. and C. Buttimore, *Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol*, 1986. 6(8):2895-902) LXSN consists of the necessary bacterial shuttle genes and a retroviral insert (Figure 1). The retroviral insert has 5' and 3' long terminal repeat (LTR) sequences for retroviral integration and a SV40 promoter controls neomycin phosphotransferase expression for use as a selectable marker with the neomycin homologue G418. Additionally, LXSN has a extended packaging signal which allows the vector to produce viral particles in an appropriate packaging cell line. (Miller, A.D. and C. Buttimore, *Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production.* Mol Cell Biol, 1986. 6(8):2895-902; Miller, A.D., *Retrovirus packaging cells.* Hum Gene Ther, 1990. 1(1):5-14). The packaging cell line is stably transfected with the viral gag pol and env genes. This system allows for

the production of replication incompetent retrovirus particles from an LXSN transfected packaging cell line. The LXSN vector can be modified by inserting a gene in the multicloning site located downstream from the 5' LTR promoter. Although the LXSN vector has proved useful for delivering the genes used in this invention, control experiments presented below suggest that other viral and non viral delivery methods may prove even more effective.

We have utilized two viral genes, the Herpes Simplex Virus (HSV) type 1 ICP-47 encoding gene York, I.A., *et al.*, *A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes.* Cell, 1994. 77(4):525-35; Rosenthal, K.L., *et al.*, *Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes.* J Virol, 1987. **61**(8):2438-47) and the human Cytomegalovirus (hCMV) US11 gene Jones, T.R., *et al.*, *Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains.* J Virol, 1995. **69**(8):4830-41; Wiertz, E.J., *et al.*, *The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol.* Cell, 1996. 84(5):769-79) to down-regulate the cytotoxic T lymphocyte (CTL) immune response to gene therapy vectors.

The LISN vector was made by cloning the HSV ICP-47 gene from HSV DNA and subcloning it into the LXSN gene therapy vector (Figure 2A). The HSV ICP-47 gene codes for a 87 amino acid immediate early HSV protein. The ICP-47 protein binds to the TAP protein of the MHC I processing and presentation pathway and inhibits its ability to transport the viral peptides into the ER (Hill, A., *et al.*, *Herpes simplex virus turns off the TAP to evade host immunity.* Nature, 1995. **375**(6530):411-5; Ahn, K., *et al.*, *Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. Embo J*, 1996. 15(13):3247-55). Without a peptide, MHC-I molecules are rapidly degraded and not transported to the cell surface (York, I.A., *et al.*, *A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T*

lymphocytes. Cell, 1994. 77(4):525-35; Fruh, K., *et al., A viral inhibitor of peptide transporters for antigen presentation.* Nature, 1995. 375(6530):415-8).

A second viral gene shown to down regulate MHC-I cell surface expression, hCMV US11, has also recently been identified and characterized. MHC-I molecules consist of a heavy chain anchored into the membrane and an associated β2 microglobulin chain. The hCMV US11 gene product has been shown to translocate the MHC-I heavy chain from the ER lumen to the cytosol where it is degraded by proteosomes (Machold, R.P., *et al., The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complex (MHC) class I heavy chains.* J Exp Med, 1997. 185(2):363-6; Jones, T.R. and L. Sun, *Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains.* J Virol, 1997. 71(4):2970-9). The LUSN vector was constructed by cloning the hCMV US11 gene from a hCMV infected cell line and subsequently subcloning it into the LXSN vector (Figure 2B).

A third vector has been constructed in a similar fashion incorporating the adenovirus GP19k encoding gene (data not shown), LGSN.(Figure 12) GP19k has also been implicated in suppression of the MHC I processing and presentation pathway and the LGSN vector was also be used in these studies.

In order to test both the LISN and the LUSN vectors to determine their effectiveness in down regulating MHC-I expression, it was necessary to construct cell lines expressing the proteins. Two murine packaging cell lines were used to generate viral supernatants. First, GPE86 cells were transfected with the vectors using a liposomal transfection method. Supernatants from the GPE86 lines were used to transduce PA317 cells and successful transfectants were identified by selection in G418 containing media. The supernatants from the PA317 cell lines were then used to transduce A375 cells. A375 is a human melanoma cell line and the above procedure resulted in 2 mixed population cell lines; A375/LISN and A375/LUSN. The same 3 step method was used to generate a A375 control cell line with an LXSN vector

incorporated A375/LXSN. Northern blot analysis confirmed the presence of LISN and LUSN RNA in the appropriate cell lines. (Figure 3). To assess MHC-I cell surface expression, a anti MHC-I monoclonal Ab, H58A and a FITC labeled ant mouse secondary Ab were used. The results were analyzed using a fluorescent activated cell sorter. The cells were not permeabolized during the Ab labeling process and thus a decrease in fluorescence indicates a decrease in cell surface MHC-I. The autofluorescence of A375 cells and the background binding of the FITC labeled secondary Ab were assessed and found to be negligible (Figure 4). The A375/LXSN cell line often showed a marked increase of cell surface MHC-I expression which has been observed as a general response to viral infection. The A375/LXSN is the more appropriate control for these experiments since the majority of gene therapy delivery methods are viral based. Both the LISN and the LUSN vectors significantly reduced the MHC-I expression from either the A375/LXSN level or the A375 level.

In order to determine if the reduction in cell surface MHC-I has the desired effect of reducing the CTL lysis of cells, a cytotoxicity assay was performed (Figure 5). This assay measures the ability of CTL's to lyse target cells by measuring the release of a stable cytosolic enzyme, lactate dehydrogenase (LDH). After incubating the vector transduced target cells with the CTL effector cells, the amount of LDH released is measured using a substrate color reaction read at O.D. 492. Five effector to target ratios were used and both spontaneous release and media background LDH were subtracted from the readings. A clonally selected T acute lymphoblastic leukemia (TALL) cell line (TALL-104, ATCC) was used as effector cells. TALL-104 cells are known to have a high specific lysis of tumor cells and thus are well suited for this assay. As predicted from the MHC-I cell surface results, A375/LXSN shows the highest specific lysis by TALL cells, followed by A375, A375/LISN, and A375/LUSN respectively. The A375 cell lines show up in Figs. 4 and 6D. 6D was a repeat of 4 and thus the numbers are slightly different. As seen in the experiment graphed in Figure 4 The 18 fold

reduction in cell surface MHC-I of A375/LUSN vrs. A375/LXSN (0.93 to 17.5 respectively) resulted in approximately a 43% reduction in TALL lysis at the 10:1 ratio. The 8 fold reduction in cell surface MHC-I of A375/LISN vrs. A375/LXSN (2.19 to 17.5) corresponded to a 32% drop in TALL lysis. These results demonstrate that the level of reduction of MHC-I on the cell surface observed is enough to reduce the cytolytic activity of CTL's. These results were consistent in all the cell lines tested. Thus, both the HSV ICP-47 gene and the hCMV US11 gene protect the LXSN gene therapy vector from the host's CTL response.

Vector construction:

Primers to the Herpes virus gene encoding ICP 47 were designed with the following characteristics: Forward primer: 24 bp complementary to the start of the gene sequence, Kosak sequence to promote ribosome binding, Hind III endonuclease restriction site, and a guanine and a cytosine nucleotide added to help stabilize the primer end and the Hind III site. Reverse primer: 24 bp complementary to the antisense sequence the end of the gene, Hind III site, and a GC clamp. PCR amplification of the gene was performed and confirmed by agarose gel electrophoresis (data not shown). Products were cloned non directionally into the PCR3® (Invitrogen) T/A cloning vector which takes advantage of A overhangs left by TAQ polymerase during the amplification. Samples were sequenced to confirm their identity (data not shown). ICP 47 was then sub cloned into the LXSN murine retrovirus backbone at the Eco RI site. Restriction digests confirmed LISN correct orientation. The same process as above was followed to generate the LUSN vector with the following differences. The primers were designed for the hCMV US11 gene and the template was purified hCMV DNA (Sigma). LISN was constructed in the following manner.

Northern analysis:

RNA was purified from the transduced mixed A375 cell lines using a total RNA purification kit (Quiagen Inc. Rneasy™). 10 μg of purified RNA was loaded onto each lane of a precast 1.25% agarose gel (FMC Bioproducts) and separated by electrophoresis. The RNA was southern blotted to .45μm pore Nylon membrane (Oncor Sure Blot™) and cross-linked using ultraviolet light. ^{32}P radiolabeled DNA probes were made for both the ICP 47 and US11 genes from non LXSN vectors to avoid LXSN contamination using a random primed DNA labeling kit (Boehringer Manheim Inc.). Probes were incubated with the membrane for 12 hours at 42 degrees in hybridization solution (Oncor Hybrizol™ I) and washed 5 minutes 2 times in 2x SSC/.1%SDS. The membrane was exposed to film and developed.

MHC I Expression assay:

Cell surface MHC I inhibition. Transduced mixed populations of A375 cells were labeled with anti-MHC I Ab (VMRD Inc.) at 0.1μg/.5×10^6 cells at 37 degrees for 1 hour. Cells were washed with PBS 2x and a 1:150 dilution of goat anti mouse FITC labeled secondary Ab (Sigma) was added for 1 hour at 37 degrees. Cells were again washed 2x and resuspended in Phenol red free/serum free Opti-MEM® (Gibco BRL) media for FACS analysis. Three samples of each were averaged and the standard deviations determined. A375 no Ab, A375 no primary, and A375 are nontransduced controls.

CTL response suppression assay:

Specific Lysis of A375 transduced target cells by TALL-104 effector cells. 5x10^3 target cells/well are aliquoted on a 96 well tissue culture plate (4 wells/data point). Effector cells are added at 10:1, 7.5:1, 5:1, 2.5:1, and 1.25:1 ratios and incubated for 7 hours. 100% lysis is determined by completely lysing a set of control wells. Cell lysis is determined by measuring the amount of a stable cytosolic enzyme (lactate dehydrogenase, LDH) released into the

well and using a color reaction (Promega Corp. Cytotox 96® assay kit). Baseline LDH levels are established and spontaneous LDH release by effector and target cells is subtracted. Extent of color reaction is determined using a 96 well plate spectrophotometer at 492 nm.

EXAMPLE 2

After constructing LISN and LUSN, an experiment was conducted as described above to determine if the genes were expressed and worked as expected within the retroviral system in other cell lines in addition to A375. Vector producer cells were made and several cell lines transduced. Northern analysis confirmed transcription of both genes in the targeted cell lines (data not shown). Cell surface MHC I was measured using a anti-MHC I monoclonal primary Ab and a FITC labeled secondary Ab. The results were measured using fluorescence activated cell (FACS) analysis (Fig. 6A-F). Mixed populations of cells transduced with the LISN or LUSN vector showed a significant reduction in cell surface MHC I as compared to the LXSN transduced control in all of the human cell lines tested. Therefore, both genes tested significantly reduced the level of MHC I cell surface expression in these cell lines.

To determine if the decrease in MHC I would lead to diminished CTL activity, a clonally selected T acute lymphocytic leukemia (TALL-104) cell line was obtained. TALL-104 is a CD8+ CTL line that shows enhanced cytotoxic effects against tumor cells in general. (R., O.C., et al., "Growth factor requirements of childhood acute T-lymphoblastic leukemia: correlation between presence of chromosomal abnormalities and ability to grow permanently in vitro", *Blood*, 77(7):1534-45 (1991). The TALL cells were tested to determine their ability to lyse the A375 and VA13 cell lines in a specific manner (Fig. 7A and B). The reduced levels of cell surface MHC I observed earlier are mimicked by the reduced levels of specific lysis by TALL cells. From this data we conclude that reduction of MHC I cell surface expression leads to a reduction in the lysis of LISN or LUSN transduced cells

by CTL's *in vitro*. All viral and cell culture materials used in these experiments are treated as biosafety level two agents in accordance with institute policy.

EXAMPLE 3

ICP 47 and US11 each reduce the expression of MHC I restricted epitopes. To determine if both genes work better than either gene does alone, two different approaches may used. First, cotransductions of two separate vectors with different selection methods would be done. Second, both genes were expressed from a single construct containing an IRES internal promoter. A single construct expressing two genes eliminates differences in RNA levels as a complicating factor. Both of the above mentioned approaches would be pursued concurrently as to avoid unforeseen problems with the relatively new IRES technology. See Figures 15, 16, 19, and 20. A vector similar to LISN would be constructed that uses hygromycin resistance as a selection method (Fig. 8). This LISH vector would then be transfected into packaging cells and the supernatant would be used to transduce target cells already containing the LUSN vector. The double transduced cells were be selected for using both G418 and hygromycin.

EXAMPLE 4

The compact genomes found in viruses led to the evolution of a system to express multiple genes off of a single RNA molecule by incorporating internal ribosome entry sites (IRES). A commercially available IRES, (Novagen pCITE-4a-c(+)) is approximately 450 bp long and has been shown to initiate translation very effectively. (Parks, G.D., G.M. Duke, and A.C. Palmenberg, "Encephalomyocarditis virus 3C protease: efficient cell-free expression from clones which link viral 5' noncoding sequences to the P3 region", *J Virol* **60**(2):376-84 (1986). The LUISN vector (Fig. 9) will be made and used to transduce target cell lines.

The level of MHC I cell surface reduction caused by either of the above models will be determined using the antibody based protocol described (Fig. 6). The specific lysis inhibition will be determined using the method described (Fig. 7).

EXAMPLE 5

Both HSV ICP 47 and hCMV US11 significantly lower the amount of cell surface MHC I and CTL specific lysis of the cell lines tested. Potential application of these genes in a gene therapy protocol would likely involve targeted vector delivery to a variety of organs and tissues. Additionally, it has been demonstrated that there are significant differences in gene expression when comparing established cell lines to primary cell cultures. To assess the function of LISN and LUSN in primary cell cultures, the MHC I transduction, expression, and cytotoxicity experiments will be repeated using selected primary cell cultures. Primary cultures from human liver, lung, kidney, and bone marrow are now commercially available and will be transduced and tested for MHC I cell surface reduction. Primary cell cultures from other tissues may also be tested.

Packaging cell lines have been established for the LISN and LUSN vectors. Supernatants from these lines will be used to transduce the target cells listed above. The transduced target cells will be selected for 2-4 weeks in G418, a neomycin homologue.

MHC I cell surface reduction will be assessed using the method described (Fig. 6). Specific lysis by TALL-104 cells will be measured as described (Fig. 7).

EXAMPLE 6

To determine if the reduction of CTL activity will result in prolonged vector survival *in vivo* will be beneficial. The potential applications of this approach are quite varied and models to address specific applications need to be made. A complicating factor concerning selection of *in vivo* models is

demonstrated in the functions of ICP 47 and US121 shown in Figure 10. ICP 47 does not function in rat cell lines, such as the 9L cell line shown here, and other rodent lines as reported elsewhere. (Ahn, K., et al., "Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47", *EMBO J*, 15(13):3247-55 (1996)) This is due to the absence of the ICP 47 binding site on the rodent version of TAP. Therefore, *in vivo* rodent models will only be used to test US11 function. ICP 47 *in vivo* function will be assessed in models where it is known to work, such as dogs as shown by the dog bone marrow cell line D17. Additionally, the complexities of *in vivo* models make it prudent to pursue several avenues in the hopes of success. The first model will express the fluorescent marker gene GFP in bone marrow cells. (Heim, R., A.B. Cubitt, and R.Y. Tsien, "Improved green fluorescence [letter]", *Nature*, 373(6516):663-4 (1995) This approach will be useful in assessing the incorporation of ICP 47/US11 immune down-regulation in the treatment of many different genetic diseases originating in blood cells. A second model will look at using the immune suppression itself as the therapeutic treatment of organ transplant and/or autoimmune disease. Recent improvements in whole organ transduction techniques makes it possible to conduct organ transplant experiments after transducing the organ with gene therapy vectors. (Shaked, A., et al., "Adenovirus-mediated gene transfer in the transplant setting. II. Successful expression of transferred cDNA in syngeneic liver grafts", *Transplantation*, 57(10:1508-11 (1994) A third model will be pursued in studying α-L-iduronidase deficiency in dogs. Shull, et. Al., Human Gene Therapy, 7:1595-1603 "Myoblast gene Therapy in Canine Mucopolysaccharidosis I: Abrogation by an Immune Response to α-L-Iduronidase" (1996).

Transduced lymphocytes are rapidly removed from the general lymphocyte population *in vivo* by both cellular and humoral immune responses. (Tripathy, S.K., et al., "Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-

defective adenovirus vectors", *Nat Med*, 2(5):545-50 (1996); Riddell, S.R., et al., "T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients", *Nat Med* 2(2):216-23 (1996)) The cellular immune response peaks at 7 days whereas the Ab response peaks at approximately 14 days. Removing the CTL response should prolong the survival of transduced lymphocytes *in vivo*. To obtain fluorescent lymphocytes, three vectors will be constructed LGSN, LGISN, and LGUSN (Fig. 11). Vector producer cells will be established and supernatants used to transduce lymphocytes.

Murine lymphocytes will be isolated, transduced, selected, and transfused back into syngeneic animals. Persistence of the fluorescent marker will be assessed by FACS analysis of periodic blood samples.

The most direct application of these immune suppression genes would be to use the immune suppression as the treatment. The appeal of this approach is to minimize the number of genes needed to transfer by making the immune suppression gene the therapeutic gene. Currently, there are several techniques that can be used to transduce whole organs such as the liver with high efficiency. Additionally, several autoimmune disorders such as certain types of diabetes, arthritis, lupus, etc. have been traced to CTL activity against self epitopes. As primary cell culture data and animal models for these diseases are available, it will be possible to test US11/ICP 47 function.

As an example of what we plan to do with this approach. There is a dog model system to treat MPS I (mucopolysaccharidosis I) using a corrected version of the canine α-L-iduronidase gene. Initial trials ran into immune response problems. This vector incorporates immune suppression into the α-L-ID vector and if it works could be used with the human α-L-ID gene to treat the disease. Fassati, A., "Insertion of two independent enhancers in the long terminal repeat of a self-inactivating vector results in high-titer retroviral vectors with tissue-specific expression", *Human Gene Therapy*, 9:2459-2468

(1998); Shull, et al., "Humoral immune response limits gene therapy in canine MPS I", *Blood*, 88(1): 377-379 (1996).

This protocol requires the construction of a vector expressing three genes. The LαSI/N and LαSU/N constructs shown in Figure 12 would satisfy the requirements. The vector will be used to transduce bone marrow cells and α-ID production will be monitored long term. Previous experiments without immune suppression genes demonstrated that about 5% of bone marrow cells express α-ID at sub-therapeutic levels long term and that the low expression levels are due in part to an immune response to the α-ID protein.

What is claimed is:

1. A nucleotide expression system for introduction of a therapeutic gene comprising:

a nucleotide sequence which encodes an immune suppression gene;

a promoter; and

a transcription termination signal, wherein said system upon transformation into recipient cells is capable of inhibiting, evading, or eliminating recipient cell immune response to said therapeutic gene.

2. The expression system of claim 1 wherein said immune suppression gene is selected form the group consisting of: SIV (Simian immunodeficiency virus) nef gene; Epstein-Barr virus BHRF1, LMP-1 and LMP-2A; adenovirus E1B/19k and E1B/55k; cowpox virus crmA and CHOhr; baculovirus p35 and IAP; molluscum contagiosum virus MC159 and MCO66L; equine herpesvirus-2 E8; rabbitpox virus SPI-1; simian virus 40 T-Ag; papilloma virus E7; cytomegalovirus US11, IE2, UL18, and US6; myxoma virus M-T5, MT-2, and M-T4; vaccinia virus E3L and K3L; and herpesvirus ICP 47 and samurai Tip.

3. The expression system of claim 1 wherein said immune response is selected form the group consisting of: cytotoxic T lymphocyte response, MHC-I, MHC-2, T helper cell, cytokines, Interleukin, and natural killer cells.

4. A recombinant viral vector for permanent introduction of a therapeutic gene in recipient cells comprising: the nucleotide expression system of claim 1 wherein said immune suppression gene is a recombinant non-native immune suppression gene.

5. The viral vector of claim 4 wherein said viral vector provides for permanent integration of said nucleotide expression system in recipient cell DNA.

6. The viral vector of claim 4 wherein said viral vector is one which can persist indefinitely in transformed cells in latent or episomal state.

7. The vector of claim 5 wherein said vector is selected from the group consisting of: retroviral virus, adeno-associated virus, Herpes virus, Lentivirus, and Epstein Barr virus.

8. The vector of claim 4 further comprising: an expression system which encodes a marker selection gene for selection of transformants.

9. The vector of claim 4 further comprising an internal ribosome entry site.

10. The vector of claim 4 wherein said selection gene is an antibiotic resistance gene.

11. The vector of claim 10 wherein said antibiotic resistance gene is selected from the group consisting of: an ampicillin resistance gene, kanamycin resistance gene, tetracycline resistance gene, zeocin resistance gene.

12. The vector of claim 4 further comprising:
a multiple cloning site

13. The vector of claim 4 further comprising: a procaryotic gene for propagation of said vector in bacteria.

14. The expression system of claim 1 or 4 further comprising a promoter selected from the group consisting of: an inducible promoter, a constitutive promoter, a tissue specific promoter and an artificial promoter enhancer.

15. The recombinant viral vector of claim 4 wherein said immune suppression gene is selected form the group consisting of: Epstein-Barr virus

BHRF1, LMP-1 and LMP-2A; adenovirus E1B/19k and E1B/55k; cowpox virus crmA and CHOhr; baculovirus p35 and IAP; molluscum contagiosum virus MC159 and MCO66L; equine herpesvirus-2 E8; rabbitpox virus SPI-1; simian virus 40 T-Ag; papilloma virus E7; cytomegalovirus US11, IE2, UL18, and US6; myxoma virus M-T5, MT-2, and M-T4; vaccinia virus E3L and K3L; and herpesvirus ICP 47 and samurai Tip.

16. The viral vector of claim 4 wherein said immune response is selected form the group consisting of: cytotoxic T lymphocyte response, MHC-I, MHC-2, T helper cell, cytokines, Interleukins, and natural killer cells, neutrophils, β-cells, plasma cells, tissue macrophages, dendritic cells, and macrophages.

17. The expression system of claim 16 wherein said immune response is MHC-I.

18. A recipient cell transformed with the vector of claim 4.

19. A method of reducing host cell immune response to introduced therapeutic genes comprising: introducing to said host cell the nucleotide vector of claim 4.

20. A method for treating MHC-I autoimmune disease comprising: administering to an animal in need of such treatment the nucleotide expression system of claim 1, wherein said immune suppression gene is one which inhibits the MHC-I immune response.

21. The method of claim 20 wherein said immune suppression gene is selected form the group consisting of: human cytomegalovirus US11 and Herpesvirus ICP 47.

22. A method of killing tumor cells comprising: introducing to said cells a nucleotide expression system of claim 1 wherein said immune suppression genes are expressed at a level such that a natural killer response is generated in the recipient host cell.

23. The method of claim 22 wherein said nucleotide expression system comprises a tumor specific promoter.

24. A plasmid retroviral vector said vector comprising the following elements: an expression system comprising an immune suppression gene selected from the group consisting of US11 and ICP 47. An expression system comprising an antibiotic resistance gene.

25. A plasmid retroviral vector, said vector comprising a nucleotide sequence of SEQ ID NO: 1, 2, 3, or 4 and the functional equivalents thereof.